INFECTION AND IMMUNITY, Dec. 1985, p. 603–609
0019-9567/85/120603-07$02.00/0

Vol. 50, No. 3

Identification and Partial Characterization of *Rickettsia tsutsugamushi* Major Protein Immunogens

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Received 19 April 1985/Accepted 20 August 1985

Strains of *Rickettsia tsutsugamushi* so far examined have either three or four quantitatively predominant proteins, which apparently are surface proteins and which range in size between 50 and 63 kilodaltons. These polypeptides also were the major immunogens detected by polyacrylamide gel electrophoresis of extracted rickettsial proteins which had been precipitated by hyperimmune rabbit sera. The major proteins from different rickettsial strains share some epitopes, as evidenced by the lack of strain specificity of the rabbit sera in the immunoprecipitation tests. However, similar experiments with a limited number of monoclonal antibodies showed that strain-specific determinants also are associated with at least the 58/60-kilodalton polypeptide. A lack of strain-specific epitopes on the rickettsial surface was indicated by our inability to detect binding of heterologous antisera to the rickettsial surface by immunoferritin labeling. Because the three major proteins of the Karp and Gilliam strains are accessible to antibody in unextracted organisms, it is possible that the exteriorly exposed epitopes of these three polypeptides are strain specific and that their common determinants are normally buried in the membrane or otherwise inaccessible. Attempts to absorb out specific antibody with intact rickettsiae gave equivocal results; however, when immune complexes formed before rickettsial extraction were examined by electrophoresis, antibody appeared to have bound strain specifically with at least the 60-kilodalton protein.

Rickettsia tsutsugamushi, the etiological agent of scrub typhus, is an obligately intracellular, gram-negative bacterium. Although there is considerable cross-reactivity among members of this species, it has been possible to divide them into a number of strains according to their antigenic specificity in standard serological and in vivo protection tests (1–3, 7, 8, 13, 14). Nonetheless, reports physically characterizing strain-specific and strain-shared antigenic activities in *R. tsutsugamushi* have been meager. In the late 1960s, a soluble, "group" antigen which was extractable from rickettsiae by sonication or ether and a residual, particulate, strain-specific antigen were described in the three most widely studied strains, Karp, Gilliam, and Kato (12, 16, 17). The antigenicity of the soluble fraction was partially destroyed by treatment with 5 mM periodate or 56°C for 30 min, while the particulate antigen was resistant to inactivation by these methods. The cross-reactive soluble antigen was precipitable with ammonium sulfate.

To describe in more detail the antigenic heterogeneity of scrub typhus rickettsiae, I began a molecular dissection of *R. tsutsugamushi*, with the aims of identifying the major protein immunogens and of determining the location of strain- and species-specific epitopes. A previous report from this laboratory (10) identified in the Karp strain three major scrub typhus rickettsial proteins which were precipitated by sera from hyperimmunized rabbits, and at about the same time Eisemann and Osterman (6) demonstrated reactivity with antirickettsial sera of material eluted from six distinct regions of polyacrylamide gels. The antigenic activity was associated with a rickettsial membrane fraction and, while there was considerable cross-reactivity among the three prototype strains examined, evidence of some strain specificity also was presented (6).

The present report extends our initial study by identifying the immunogenic polypeptides of three additional strains of *R. tsutsugamushi* as detected in a system quite different from that used by Eisemann and Osterman (6) and presents the results of initial studies on the strain specificity of these immunogens. The evidence suggests that individual polypeptides contain both strain-specific and strain-shared epitopes and that group-reactive epitopes are not exposed on the surface of intact rickettsiae.

MATERIALS AND METHODS

Rickettsiae. The Gilliam and Kato strains used were from the 143rd and 45th passage levels, respectively, in specific-pathogen-free embryonated chicken eggs (SPAFAS, Inc., Norwich, Conn.). The Karp stain was either from the 107th egg passage level or a seed obtained from J. V. Osterman as a plaque-purified clone. The JC472B isolate of *R. tsutsugamushi* was obtained from an Indian gerbil in Pakistan (15) and has been passaged three times in mice and nine times in eggs. Rickettsiae routinely were prepared as 20% (wt/vol) yolk sac suspensions in brain heart infusion broth and stored at −70°C.

Cell cultures. Rickettsiae were grown in either primary chicken embryo cell, L cell, or Vero cell cultures. The choice of cell type was based on convenience; *R. tsutsugamushi* replication rates and polypeptide profiles are indistinguishable in the three (unpublished experiments). Tissue culture medium was Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) with half-strength vitamins and amino acids and supplemented with 10% fetal calf serum and 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.3.

Radiolabeled rickettsial proteins. Cells in suspension were infected as described previously (11) and cultured in 60-mm dishes at 34°C in an atmosphere of 5% CO_2 in air for a total of 5 days to permit optimal rickettsial growth, which was monitored in parallel slide cultures (11). On day 2 after infection, the cells were treated with 1 μg of emetine per ml, which inhibited host-cell protein synthesis during the subse-

quent 24 h by 60%. The next day, the growth medium was changed to leucine-free medium (minimal essential medium [Eagle]; Flow Laboratories, Inc., Mclean, Va.) containing 1 μg of emetine per ml, and the cultures were further incubated for 4 to 6 h. After this time, the medium was changed to fresh leucine-free medium which also contained 1 μg of emetine per ml, 3% dialyzed fetal calf serum (GIBCO), and 20 to 50 μCi of [^{3}H]leucine (65 mCi/mmol, SchwarzMann, Orangeburg, N.Y.; or 130 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) per ml. Incubation continued for 18 to 20 h, during which time protein synthesis relative to that in untreated controls was inhibited by over 90%. The infected cells were harvested in a manner calculated to optimize the recovery of nondegraded, cell-associated rickettsiae. The cells were washed free of released rickettsiae either before or after gently scraping them from the culture dishes and were quickly frozen in sucrose-PG-Mg buffer (218 mM sucrose, 10 mM potassium phosphate buffer [pH 7], 5 mM potassium glutamate, 10 mM $MgCl_2$) in a dry ice-ethanol bath and held at −70°C. The emetine treatment did not affect the rate of scrub typhus rickettsial replication under the conditions described (unpublished data). The specificity of the radiolabel was assured by the following tests. Similarly treated, mock-infected cultures were prepared with each lot of radiolabeled rickettsiae, and electrophoretic analysis of these extracts revealed little or no radiolabeled host protein (10). Moreover, the incorporation of leucine into polypeptides under these conditions was markedly reduced by the presence of 20 μg of chloramphenicol per ml but not by 100 μg of streptomycin per ml, an antibiotic to which *R. tsutsugamushi* is resistant (10).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A standard Laemmli system employed 13% acrylamide–0.52% diallytartardiamide slab gels in tris-glycine-SDS buffer (10). For analysis of rickettsial proteins, rapidly thawed, radiolabeled, infected cells were concentrated approximately 10-fold by centrifugation at 13,000 × *g* for 2 min in a microcentrifuge to pellet the rickettsiae and by suspension in hot sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 5 mM phosphate buffer [pH 7], 5 to 100 μM phenylmethylsulfonyl fluoride). Samples were held in a boiling water bath for 3 to 5 min before loading onto the gels. After electrophoresis at 90 to 100 V, the gels were fixed in trichloroacetic acid (TCA) and fluorographed by the method of Bonner and Laskey (4). ^{14}C-labeled molecular weight markers were obtained from Amersham.

Protein extraction. Radiolabeled, infected cells were centrifuged to sediment the rickettsiae which were then suspended in detergent and incubated for 1 h at 22 to 24°C. After centrifugation at 13,000 × *g* for 2 min in the microcentrifuge, the pellet fractions were resuspended in the original volume of the original detergent, and both the pellet and supernatant fractions were analyzed for total radioactive counts per minute and TCA-precipitable counts per minute. (Since emetine inhibited host protein synthesis by only 90% [above], host proteins may account for a small proportion of these counts per minute.) When the fractions were to be electrophoresed, they were first mixed with an equal volume of double-strength sample buffer and heated in a 100°C water bath or, alternatively, acetone precipitated and heated in sample buffer.

RIP. Immune sera, prepared in rabbits by 8 to 12 injections of rickettsia-infected mouse livers and spleens, were described in earlier studies (W. T. Walsh, Ph.D. thesis, University of Maryland, Baltimore, 1975). Control sera were taken from the same rabbits just before the first inoculation. For routine radioimmune precipitation (RIP) rickettsial proteins were extracted as described above with 1% Triton X-100–0.5% sodium deoxycholate (DOC)–0.5 M NaCl (TX-DOC-NaCl). The extracts (13,000 × *g* supernatant fractions) were mixed with equal volumes of 40% serum and held overnight at 4°C, and the immune complexes were collected with fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). After thorough washing in TX-DOC, the antigen-antibody complexes were removed from the Pansorbin by heating in sample buffer in a boiling water bath for 3 min. The *S. aureus* cells were separated by an additional centrifugation. Absorption of the sera with L cells did not alter the immunoprecipitation results with L cell-grown rickettsiae, and rickettsial proteins radiolabeled in primary chicken embryo cells, L cells, and Vero cells were immunoprecipitated similarly.

Periodate treatment of rickettsiae. For infectivity and fluorescent-antibody studies, 20% yolk sac suspensions of rickettsiae were centrifuged through Renografin (E. R. Squibb & Sons, Princeton, N.J.) to remove most of the contaminating yolk material before periodate treatment (11). (This procedure resulted in up to 100% rickettsial recovery.) The rickettsiae were then washed by centrifugation and suspended in periodate-containing 10 mM phosphate buffer (pH 7.2) plus 10 mM $MgCl_2$ or in AP buffer from which the glucose had been omitted (AP − glucose consists of 10 mM potassium phosphate buffer [pH 7.2], 5 mM potassium glutamate, 45 mM NaCl, 62.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$). The periodate concentrations varied and are given below. After incubation at 20 to 24°C for 30 min in the dark, the rickettsiae were again washed by centrifugation through Renografin and suspended in tissue culture medium for infectivity studies or in AP buffer for indirect immunofluorescence assay.

For periodate experiments requiring radiolabeled rickettsiae, infected radiolabeled cells were passed twice through a 27-gauge needle, which disrupted any remaining intact cells (unpublished observations), and then also were centrifuged through Renografin. The method of host-cell breakage, monitored by light and electron microscopy, was deliberately chosen to minimize formation of host membrane vesicles which might trap rickettsiae and render them inaccessible to periodate (unpublished observations). After partial purification with Renografin, the rickettsiae were washed by centrifugation and treated with differing concentrations of periodate as above.

Immunoferritin labeling of rickettsiae. Pellets (13,000 × *g*) of frozen and thawed rickettsia-infected Vero cells were suspended in sucrose-PG-Mg buffer and mixed with an equal volume of diluted rabbit hyperimmune or nonimmune (control) sera and incubated for 30 min at 34°C. After two washes by centrifugation, the rickettsiae were similarly incubated with ferritin-conjugated goat antisera prepared against rabbit globulins (anti-immunoglobulin A, -immunoglobulin G, and -immunoglobulin M; Cappel Laboratories, Cochranville, Pa.). After another wash to remove unbound conjugate, the rickettsiae were suspended in sucrose-PG-Mg buffer and fixed for electron microscopy (11).

Neutralization of rickettsial infectivity. Neutralization of rickettsial infectivity has been described in detail previously (11). Briefly, rickettsiae were incubated in given concentrations of immune serum for 30 min at room temperature and then added to an equal volume of suspended Vero cells. After a 60-min incubation at 34°C to permit maximum infection, the cells were washed and allowed to settle on glass slides. After fixation and staining with Giemsa stain,

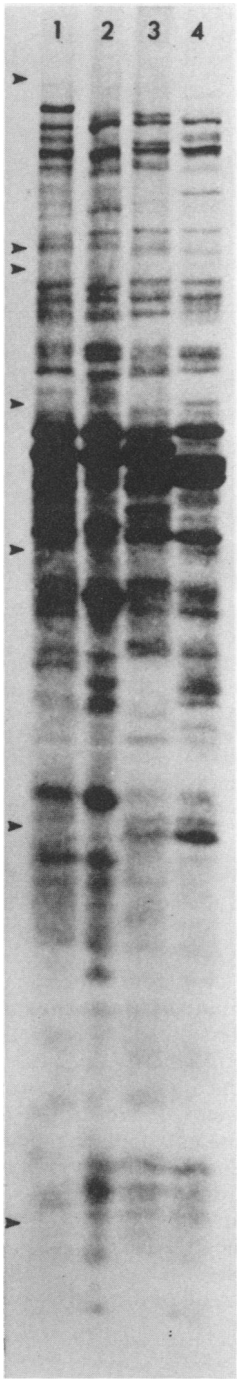

FIG. 1. SDS-PAGE of four strains of *R. tsutsugamushi*: JC472B (lane 1), Karp (lane 2), Gilliam (lane 3), and Kato (lane 4). Arrows indicate migration of molecular size markers (from top to bottom: myosin, 200 kDa; phosphorylase *b*, 100 kDa and 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and lysozyme, 14.3 kDa). (Reprinted from reference 10 with permission from Academic Press, Inc.)

the average number of cell-associated rickettsiae per cell was assessed by counting replicate samples of 100 cells each.

RESULTS

Identification of major proteins. As described earlier (10), the quantitatively major proteins of *R. tsutsugamushi* Karp, Gilliam, and JC472B migrate on SDS-polyacrylamide gels according to apparent molecular sizes of about 63, 60, and 50 kilodaltons (kDa) (Fig. 1). The Kato strain also has major proteins with apparent molecular sizes of 63 and 50 kDa, but instead of a 60-kDa polypeptide has two slightly faster migrating ones with apparent molecular sizes of 58 and 57 kDa (Fig. 1). Previously reported experiments (10) showed that the 63-, 60-, and 50-kDa proteins of strain Karp were the predominant ones immunoprecipitated by hyperimmune rabbit sera.

RIP of rickettsial polypeptides. Preparative to extensive RIP experiments, the relative efficacies of several detergents in extracting scrub typhus rickettsial proteins and in allowing their precipitation by immune sera were determined. Table 1 compares the effects of the relatively mild nonionic detergent Triton X-100 with the stronger anionic detergent SDS and with the dipolar ionic Zwittergent (Calbiochem-Behring). Virtually all the rickettsial protein was released by 0.5% SDS, by the addition of 0.5 M NaCl to Triton X-100, and by 2% Zwittergent. However, as the effective concentration of SDS increased, so did the RIP of the extracted proteins decrease, undoubtedly due to protein denaturation. (Triton X-100 competes with SDS and thus lowers the effective SDS concentration [5].) The enhancing effect of NaCl in extracting proteins while not altering the RIP in Triton X-100 plus DOC was dramatic and is discussed elsewhere (9).

These results indicated which detergents might be useful in preparing rickettsial extracts for RIP. To explore this further, the qualitative effects on RIP of several detergents which extracted at least some of each of the rickettsial proteins were determined by SDS-PAGE of the immunoprecipitates (Fig. 2). Similar to the Karp strain, the 63-, 60-, and 50-kDa proteins of strain Gilliam also were immunoprecipitated. Minor immunoprecipitated bands appear in some of

TABLE 1. Effect of detergents on release and immunoprecipitation of rickettsial proteins[a]

Detergent[b]	% Rickettsial protein cpm	
	Released[c]	Immunoprecipitated[d]
1% Triton X-100	43	71
1% Triton X-100 + 0.5% DOC	45	78
1% Triton X-100 + 0.5% DOC + 0.5 M NaCl	99	78
0.1% SDS	73	63
0.1% SDS + 1% Triton X-100	63	78
0.1% SDS + 1% Triton X-100 + 0.5 M NaCl	100	20
0.5% SDS	95	3
0.5% SDS + 1% Triton X-100	90	67
2% Zwittergent	87	91
2% Zwittergent + 0.5 M NaCl	94	100

[a] Karp or Gilliam rickettsiae were incubated for 1 h at 22 to 24°C for extraction; 13,000 × *g* supernatant fractions then were mixed with homologous antiserum in the same detergent (except for omission of any NaCl) for RIP. The two rickettsial strains reacted similarly.

[b] In water.

[c] TCA-precipitable counts per minute in 13,000 × *g* supernatant fraction as percentage of total TCA-precipitable counts per minute in the sample.

[d] Counts per minute in immune complexes as percentage of total TCA-precipitable counts per minute in extract.

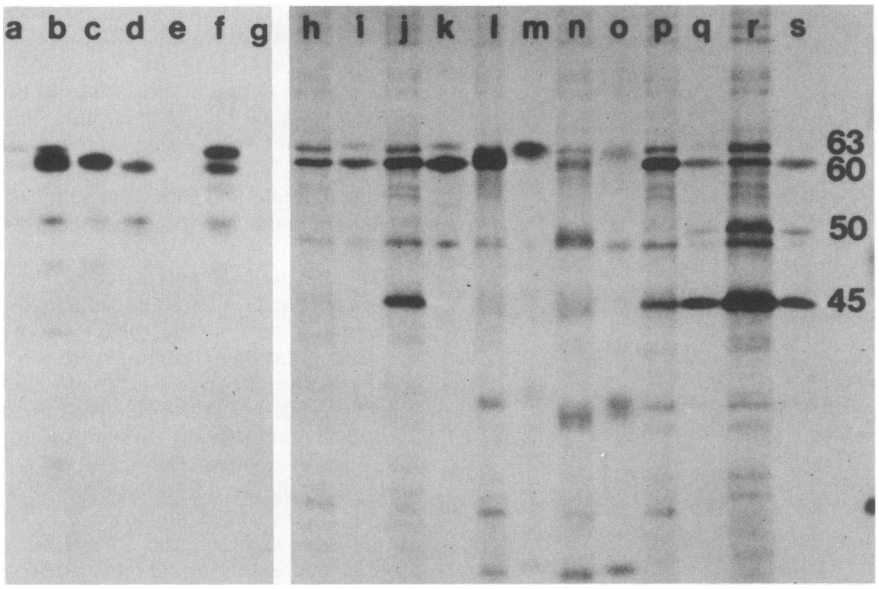

FIG. 2. SDS-PAGE of *R. tsutsugamushi* proteins extracted and immunoprecipitated in different detergents. Lanes a through g: Strain Karp rickettsial proteins extracted and immunoprecipitated in 1% Triton X-100 (a), 1% Triton X-100 plus 0.1% SDS (b), 1% Triton X-100 plus 0.5% SDS (c), 0.1% SDS (d), 0.5% SDS (e), 1% Triton X-100 plus 0.5% DOC (f and g). The serum used in the immunoprecipitations was hyperimmune rabbit and anti-Karp (lanes a through f) or serum from the same rabbit taken before immunization (lane g). Each of the extracts, which were electrophoresed on a separate gel, contained the 63-, 60-, and 50-kDa polypeptides (not shown). Lanes h through s: Extracts (h, j, l, n, p, r) and immunoprecipitates (i, k, m, o, q, s) of strain Gilliam rickettsial proteins treated in 1% Triton X-100 plus 0.5% DOC with (j and k) or without (h and i) 0.5 M NaCl; 1% Triton X-100 plus 0.1% SDS with (n and o) or without (l and m) 0.5 M NaCl; and 2% Zwittergent with (r and s) or without (p and q) 0.5 M NaCl. The serum used in the immunoprecipitations was hyperimmune rabbit anti-Gilliam. Numbers indicate positions of 63-, 60-, and 50-kDa proteins. The 45-kDa band is a conformational variant of the 60-kDa polypeptide (9).

the test systems; their significance has not been determined yet. Of the detergents tested, only Triton X-100 plus DOC supported the immunoprecipitation of the 63-, 60-, and 50-kDa polypeptides of both Karp and Gilliam, the antigenicity of the Gilliam 60-kDa protein and the Karp 63-kDa protein appearing to be most susceptible to SDS denaturation. Therefore, further characterization of the *R. tsutsugamushi* antigens has been done under as mild conditions as possible, using TX-DOC-NaCl.

Specificity of extracted proteins. To determine which polypeptides are immunoprecipitated in the two other strains shown in Fig. 1 and to see whether hyperimmune rabbit sera could distinguish the polypeptides extracted from different strains, RIP was done with extracted proteins from the Karp, Gilliam, Kato, and JC472B strains with anti-Karp, anti-Gilliam, or anti-Kato serum. The experiment shown in Fig. 3 demonstrates first that the 63-, 60-, and 50-kDa proteins of JC472B also react with immune sera in this test and that the quantitatively predominant proteins (63-, 58-, 57-, and 50-kDa polypeptides) of Kato likewise are its major immunogens. Second, there was little evidence of strain specificity; all of the immunogenic proteins were precipitated by each of the sera, demonstrating that group reactivity resides in each of the polypeptide species. In an attempt to detect subtle differences in the reactivity of homologous and heterologous pairs, the total counts per minute precipitated in each case was analyzed (Table 2). With one exception, this method confirmed the lack of strain specificity apparent from the fluorographs; each of the antigens was precipitated to the same extent by all of the antisera, except for the slight

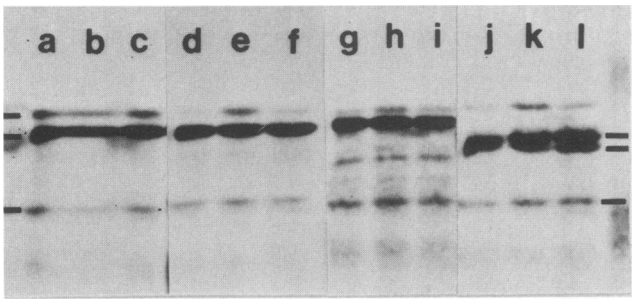

FIG. 3. Lack of strain specificity in the immunoprecipitation of extracted *R. tsutsugamushi* proteins by hyperimmune rabbit sera. Protein extracts were prepared from strains JC472B (lanes a through c), Karp (lanes d through f), Gilliam (lanes g through i), and Kato (lanes j through l) and treated with antisera prepared against strains Karp (a, d, g, j) Gilliam (b, e, h, k), or Kato (c, f, i, l). Lines indicate positions of the 63-, 60-, 58-, 57-, and 50-kDa polypeptides (from top to bottom, respectively).

TABLE 2. Immunoprecipitation of rickettsial proteins by homologous and heterologous antisera[a]

Antiserum	Antigen (cpm immunoprecipitated)[b]			
	JC472B	Karp	Gilliam	Kato
Anti-Karp	**100**	**100**	90.8 ± 6.0	76.9 ± 2.4
Anti-Gilliam	94.0 ± 2.7[b]	97.5 ± 5.8	**100**	81.2 ± 8.3
Anti-Kato	102.7 ± 1.9	101.4 ± 11.0	103.0 ± 17.4	**100**

[a] Differences in activity among antisera to Karp, Gilliam, and Kato were not statistically significant (paired *t* test, $P > 0.05$).

[b] In each experiment, for each antigen, the counts per minute precipitated by homologous antiserum were normalized to equal 100. The other values show the percentage of counts per minute of a given antigen precipitated by the homologous antiserum (mean of three experiments ± standard error).

decrease in Kato antigen precipitated by anti-Karp and anti-Gilliam sera.

These findings, however, did not rule out the additional presence of strain-specific epitopes, since reaction of antibody with just one determinant should be sufficient to precipitate the entire molecule. RIP with a limited number of monoclonal antibodies performed to assess this showed that (i) the 58-kDa protein of Kato shares epitopes with the 60-kDa proteins of Karp and Gilliam, (ii) Karp, Gilliam, and Kato 58/60-kDa proteins each have unique, noncross-reactive epitopes as well as common ones, and (iii) the Karp and Gilliam 60-kDa proteins share an epitope which is not on the Kato 58-kDa polypeptide. The single available monoclonal ascitic fluids against either the 63- or 50-kDa protein were nonspecific (Table 3).

Specificity of surface antigens. The hyperimmune sera used do have the capacity to distinguish scrub typhus rickettsial strains in another test, neutralization of infectivity (11). Although the molecular target(s) of this specific reaction is not yet known, it must be on the rickettsial surface, i.e., some strain-specific epitope(s) must be exposed on the exterior of the rickettsiae. In fact, when the binding of hyperimmune rabbit serum to intact Karp or Gilliam rickettsiae was examined by immunoferritin labeling, no nonspecific reaction was observed: anti-Gilliam serum bound only to Gilliam and not to Karp rickettsiae, and anti-Karp serum attached exclusively to Karp (data not shown). Thus, we could detect no cross-reactive antigenic sites accessible on the surface of intact organisms of these two stains with these reagents. (Note, however, that while rickettsiae washed in sucrose-PG-Mg buffer retained their major proteins, it is quite possible that some other surface components were lost.)

I have shown that the 63-, 60-, and 50-kDa proteins of unextracted Karp rickettsiae are accessible to antibody (B. Hanson, manuscript in preparation). To determine which, if any, of the major protein immunogens have regions exposed on the rickettsial surface which might be strain specific, two approaches were taken. First, hyperimmune rabbit serum was absorbed with intact rickettsiae (11) in an attempt to remove antibodies to surface components, and the reaction of the absorbed serum with extracted proteins was assessed by RIP. SDS-PAGE of the immunoprecipitates revealed no loss of serum activity against any particular polypeptide; prior treatment of anti-Karp serum with either Karp or Gilliam rickettsiae did not prevent it from reacting with the 63-, 60-, or 50-kDa protein of either strain (not shown). Quantitative analysis suggested that absorption of anti-Karp serum with intact Karp rickettsiae removed some of its capacity to precipitate Karp proteins, although there was considerable variation from experiment to experiment and the difference was not statistically significant (data not shown). Absorption with Karp had no effect on the precipitation of Gilliam antigens, nor did absorption with Gilliam influence the results with either Karp or Gilliam proteins. The absorption procedure did remove neutralizing activity from the sera (11). My inability to clearly demonstrate by this method strain-specific surface determinants on the polypeptide antigens could be due to a predominance in the polyclonal serum of antibodies to common epitopes.

TABLE 3. Specificity of monoclonal antibodies to *R. tsutsugamushi*

Monoclonal antibody[a]	Immunizing strain	Specificity[b]		
		Karp	Gilliam	Kato
K1E106	Karp	60	–[c]	–
K13F88	Karp	60	–	–
G3C51	Gilliam	–	60	–
Kt12H122	Kato	–	–	58
G7F91	Gilliam	60	60	–
Kt6G129	Kato	60	60	58
G6E11	Gilliam	63	63	63
KCH47	Karp	50	50	50

[a] Original designations of C. Eisemann.
[b] Polypeptide immunoprecipitated from detergent-extracted rickettsiae (molecular size in kDa).
[c] No polypeptide immunoprecipitated.

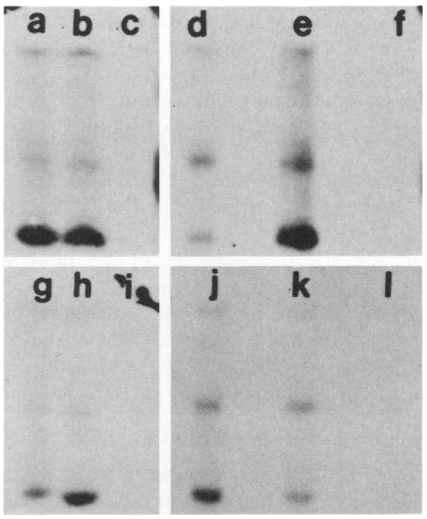

FIG. 4. Binding of antirickettsial antibody to *R. tsutsugamushi* Karp and Gilliam polypeptides before or after extraction. Gilliam polypeptides are shown after extraction in TX-DOC-NaCl followed by treatment with anti-Karp (lane a), anti-Gilliam (lane b), or normal rabbit (lane c) serum, and after treatment of intact organisms with anti-Karp (lane d), anti-Gilliam (lane e), or normal rabbit (lane f) serum followed by extraction of immune complexes. Karp polypeptides are shown after extraction in TX-DOC-NaCl followed by treatment with anti-Karp (lane g), anti-Gilliam (lane h), or normal rabbit (lane i) serum, and after treatment of intact organisms with anti-Karp (lane j), anti-Gilliam (lane k), or normal rabbit (lane l) serum followed by extraction of immune complexes. For reference, note positions in lane a of faint 63- and 50-kDa band and the heavy 60-kDa band at the bottom of the figure. (The heat-modifiable 60-kDa polypeptide migrated ahead of the 50-kDa protein because, in the experiment shown, the samples were not boiled before electrophoresis [9]; heating or not heating the samples before electrophoresis did not affect the results other than the polypeptide migration pattern [replicate experiments not shown].)

In a second method to determine if any of the polypeptides have strain-specific, outwardly accessible epitopes, washed but otherwise untreated rickettsiae were incubated with immune serum and thoroughly washed free of unbound antibody before the antigen-antibody complexes were extracted with TX-DOC-NaCl and collected with Pansorbin (Fig. 4). Under these conditions, at least the 60-kDa proteins now appeared to react with immune serum strain specifically: while the extracted Gilliam 60-kDa polypeptide was precipitated by anti-Gilliam and anti-Karp serum about equally, before extraction it bound much more to homologous anti-Gilliam serum than to the heterologous anti-Karp serum. Similarly, the extracted Karp 60-kDa polypeptide reacted a little more strongly with the anti-Gilliam serum than with the anti-Karp serum, but the unextracted protein associated less well with the heterologous antiserum than

with anti-Karp. (Much of the 63-kDa protein probably was lost during the extensive washing of the rickettsiae in sucrose-PG buffer without $MgCl_2$ [9].)

Effect of periodate on immune serum reactions. To determine a possible contribution of carbohydrate to rickettsial antigenic activity, the effect of periodate on the reactions described above was examined. At the highest concentration (0.1 mM) which could be used without inactivating the organisms, periodate pretreatment of rickettsiae did not alter the extent or the strain specificity of the neutralization of infectivity by immune serum (data not shown). Moreover, rickettsial treatment with higher concentrations of periodate (up to 10 mM) had no effect on the extent of the reactions or the degree of strain specificity of the sera when tested by an indirect immunofluorescence assay or RIP (data not shown).

DISCUSSION

SDS-PAGE of intrinsically radiolabeled *R. tsutsugamushi* proteins in slab gels has provided a means of readily distinguishing the four scrub typhus rickettsial strains examined (10). The quantitatively predominant rickettsial proteins, ranging in apparent molecular size from 50 to 63 kDa, also were the major immunogens detected by immunoprecipitating extracted proteins with hyperimmune rabbit sera. While the three major proteins of Karp and Gilliam migrate similarly on SDS gels, the Kato strain is distinguished by possessing four predominant polypeptides rather than three; the 58-kDa protein of Kato corresponds to the 60-kDa proteins of Karp and Gilliam, since they all react with the same monoclonal antibody. The major proteins probably are associated with the rickettsial outer membrane (B. Hanson, manuscript in preparation).

While three (Karp and Gilliam) or four (Kato) immunoprecipitation bands were regularly found under the standard conditions (extraction with TX-DOC-NaCl), in the presence of SDS some faster-migrating polypeptides were also immunoprecipitated from Karp and Gilliam extracts, perhaps due to the uncovering of cryptic antigenic sites, to protein fragmentation, or to anomalous polypeptide migration. SDS was not routinely included in the extraction buffer, however, because it apparently prevents immunoprecipitation of the Gilliam 60-kDa protein and of the Karp 63-kDa protein even in the lowest concentration tested. In addition, occasionally, one or more extra immunoprecipitated bands appeared in the gel near the 50-kDa protein. These have not yet been characterized. Using a method with considerably less resolving power than mine, Eisemann and Osterman (6) reported finding six antigenic fractions after electrophoresing *R. tsutsugamushi* extracts on SDS gels and performing enzyme-linked immunosorbent assays on eluted fractions. The two major antigenic peaks, numbered 2 and 3, corresponded to the two major peaks of radioactivity in amino acid-labeled rickettsial membrane extracts, but antigenic fractions also were found in regions of the gels corresponding both to greater and lesser molecular sizes. Using monoclonal antibodies prepared and characterized by C. Eisemann, I found that the antibodies specific for their antigen number 2 reacted with either the 63- or 60-kDa protein in our system and that an antibody which reacts with antigen number 3 in their system immunoprecipitated the 50-kDa protein in my laboratory. Thus, it appears that the two major protein peaks detected by Eisemann and Osterman (6) correspond to the three major proteins of Karp and Gilliam I identified in slab gels.

The occurrence of both strain-specific and common epitopes on scrub typhus rickettsial polypeptides was detected with hyperimmune rabbit sera and monoclonal antibodies. The extensive cross-reactivity among proteins from related strains was not surprising, since these proteins must have placed upon them some of the same restrictions (e.g., association with the outer membrane, biological function?) which could lead to their conservation.

Several lines of evidence point to the location of strain-specific antigenic determinants on the rickettsial outer surface. First, antibody-mediated neutralization of *R. tsutsugamushi* attachment or penetration of target cells is strictly strain specific (11). Moreover, no binding of immunoglobulin to surface-exposed sites on intact heterologous rickettsiae was detectable by the immunoferritin labeling technique, while the homologous controls were strongly positive. Third, antibody was found to bind strain specifically to the 60-kDa proteins of Karp and Gilliam when its interaction was apparently limited to external rickettsial sites. Greater variation in the surface-exposed portions of the antigenic molecules implies that alterations in those regions do not affect the overall structure enough to significantly inhibit their functions, which as yet are unknown. Future experiments with monoclonal antibodies will explore this point further and should, among other things, permit us to identify the epitopes associated with infectivity.

The results reported here may be compared with other studies of common and unique antigenic determinants possessed by scrub typhus rickettsial strains. Depending on the combination of rickettsial strain and mouse antirickettsial serum, some cross-reactivity occurred among strains Karp, Gilliam, and Kato in each of the six antigenic fractions studied by Eisemann and Osterman (6), but the patterns suggested some strain specificity too, particularly of the antigen fractions other than numbers 2 and 3. The relatively poor resolution of their gel system makes it uncertain that each of the fractions contained only one molecular species (see above), but the basic conclusions from their data are not inconsistent with mine.

My results also are compatible with those of Shishido and co-workers (16, 17) and Kobayashi et al. (12), who found that Karp, Gilliam, and Kato antigens extracted by sonication or ether were cross-reactive in the complement fixation test, while the antigens remaining in the particulate fraction were strain specific. Using sonication and centrifugation procedures analogous to those of Shishido and co-workers (16, 17), I found that nearly all of the 63-kDa protein and various amounts of the 50- and 60-kDa proteins were in the supernatant fraction which the earlier authors referred to as "soluble" antigen (unpublished data). Similarly, ether treatment based on the method of Kobayashi et al. (12) released all of the 63-kDa protein and smaller amounts of the 50- and 60-kDa polypeptides into the soluble fraction (unpublished data). My observations suggest the possibility that the antigens in the soluble fraction were cross-reactive because their normally buried common epitopes were exposed by the treatment of the rickettsiae with sonication or ether. By the same token, in the particulate rickettsial shells, antibody access might be limited to the exterior, variable epitopes, and therefore this fraction would appear to be antigenically strain specific. Alternatively, the Japanese investigators may have been dealing with antigenic determinants not identified in my experiments.

Speculation on the role of the protein immunogens described here (and of other scrub typhus rickettsial immunogens) in the establishment of protective immunity clearly would be premature. It is interesting (and of obvious importance) that long-lasting immunity to scrub typhus is strain

specific (13, 18). Whether this is related to the occurrence of a particular specific protective antigen, to the apparent surface location of specific epitopes, or to some other factor cannot be assessed with my present information. My results do suggest that immune mechanisms involving reaction with rickettsial antigens presented in situ in membranes, e.g., in intact organisms (or inserted into host membranes?), might be strain specific. Our understanding of these issues must await further studies both of the rickettsial antigens and of the mechanisms by which an immune host suppresses a rickettsial infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17743 from the National Institute for Allergy and Infectious Diseases.

I thank Kathryn Sheridan for her superb assistance in the laboratory, David Silverman for his patient help with the electron microscopy, and Christine Eisemann for her most generous gift of monoclonal antibodies.

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